RESEARCH ARTICLE

Proteins Needed to Activate a Transcriptional Response to the Reactive Oxygen Species Singlet Oxygen

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ABSTRACT **Singlet oxygen ($^1O_2$) is a reactive oxygen species generated by energy transfer from one or more excited donors to molecular oxygen. Many biomolecules are prone to oxidation by $^1O_2$, and cells have evolved systems to protect themselves from damage caused by this compound. One way that the photosynthetic bacterium *Rhodobacter sphaeroides* protects itself from $^1O_2$ is by inducing a transcriptional response controlled by ChrR, an anti-σ factor which releases an alternative sigma factor, σ^E, in the presence of $^1O_2$. Here we report that induction of σ^E-dependent gene transcription is decreased in the presence of $^1O_2$ when two conserved genes in the σ^E regulon are deleted, including one encoding a cyclopropane fatty acid synthase homologue (RSP2144) or one encoding a protein of unknown function (RSP1091). Thus, we conclude that RSP2144 and RSP1091 are each necessary to increase σ^E activity in the presence of $^1O_2$. In addition, we found that unlike in wild-type cells, where ChrR is rapidly degraded when $^1O_2$ is generated, turnover of this anti-σ factor is slowed when cells lacking RSP2144, RSP1091, or both of these proteins are exposed to $^1O_2$. Further, we demonstrate that the organic hydroperoxide *tert*-butyl hydroperoxide promotes ChrR turnover in both wild-type cells and mutants lacking RSP2144 or RSP1091, suggesting differences in the ways different types of oxidants increase σ^E activity.**

IMPORTANCE **Oxygen serves many crucial functions on Earth; it is produced during photosynthesis and needed for other pathways. While oxygen is relatively inert, it can be converted to reactive oxygen species (ROS) that destroy biomolecules, cause disease, or kill cells. When energy is transferred to oxygen, the ROS singlet oxygen is generated. To understand how singlet oxygen impacts cells, we study the stress response to this ROS in *Rhodobacter sphaeroides*, a bacterium that, like plants, generates this compound as a consequence of photosynthesis. This paper identifies proteins that activate a stress response to singlet oxygen and shows that they act in a specific response to this ROS. The identified proteins are found in many free-living, symbiotic, or pathogenic bacteria that can encounter singlet oxygen in nature. Thus, our findings provide new information about a stress response to a ROS of broad biological, agricultural, and biomedical importance.**

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Different classes of reactive oxygen species (ROS) are formed as a consequence of either electron or energy transfer reactions to molecular oxygen (1, 2). Singlet oxygen ($^1O_2$) is a ROS generated by energy transfer from one or more donors to oxygen (2–6). $^1O_2$ has the potential to irreversibly damage DNA, proteins, or fatty acids (6–8); therefore, it is not surprising to find that cells mount stress responses to protect themselves from the deleterious effects of this compound (2, 4–6, 9). One way that cells protect themselves against $^1O_2$ is by inducing a transcriptional response in the presence of this ROS (2, 6). Despite the ability of $^1O_2$ to induce gene expression in prokaryotic and eukaryotic cells (2, 6), we lack information on how these transcriptional responses are activated.

We study the transcriptional response to $^1O_2$ in the photosynthetic bacterium *Rhodobacter sphaeroides* (2). In this and other photosynthetic cells, $^1O_2$ is formed at significant levels as a consequence of energy transfer from light-excited photopigments to oxygen (2, 5, 6, 10). When $^1O_2$ is formed in *R. sphaeroides*, the resulting transcriptional response is controlled by ChrR, an anti-σ factor which normally binds its cognate alternative sigma factor, σ^E, and prevents it from activating target genes (2, 4, 11). When $^1O_2$ is generated, it promotes dissociation of σ^E-ChrR complexes (11), releasing σ^E so that it can bind RNA polymerase and activate transcription of target genes (12). Some σ^E target genes are needed for viability in the presence of $^1O_2$ (9, 13, 14), illustrating the important role of the transcriptional response to this damaging molecule.

Previous studies indicate that σ^E directly regulates ~15 genes, some of which are conserved across diverse species of photosynthetic and nonphotosynthetic bacteria (9). Genes in this core σ^E regulon include the structural genes for the two master regulators (*rpoE-chrR*; RSP1092-RSP1093), for RSP2143-RSP2144 (which encode homologues of deoxyribopyrimidine photolyase and bacterial cyclopropane fatty acyl synthase, respectively), and for RSP1091-RSP1087 (an operon divergently transcribed from *rpoE-chrR* encoding proteins of unknown function). The other direct σ^E target genes are part of an extended regulon, as they were not

predicted to be part of the σ^E-dependent transcriptional response outside photosynthetic bacteria (9). Members of this extended σ^E regulon include *rpoH*$_{II}$ (RSP0601, which encodes one of two *R. sphaeroides* heat shock sigma factors). The existence of ChrR and of σ^E target genes across the bacterial phylogeny predicts that aspects of this transcriptional response are conserved in diverse photosynthetic and nonphotosynthetic organisms (9). While $^1O_2$ formation induces the expression of many genes, several encode proteins of unknown or only predicted function (including RSP1091-RSP1087, RSP2144, and RSP1409). Elucidating how these uncharacterized gene products function may give insight into how the signal cascade is initiated upon $^1O_2$ exposure.

In this report, we show that mutants in two genes in the core σ^E regulon (RSP2144 and RSP1091) are deficient in the full activation of σ^E-dependent gene transcription and in the rapid turnover of ChrR that occurs when wild-type cells are exposed to $^1O_2$. We also show that the organic hydroperoxide *tert*-butyl hydroperoxide (*t*-BOOH), which increases σ^E activity in both *R. sphaeroides* and the nonphotosynthetic bacterium *Caulobacter crescentus* (11, 15), promotes ChrR turnover in wild-type cells as well as in strains lacking either RSP2144 or RSP1091. This finding predicts that there are differences in how $^1O_2$ and *t*-BOOH increase σ^E activity. We present a model for how RSP2144 and RSP1091 promote ChrR degradation in the presence of $^1O_2$ and why *t*-BOOH and $^1O_2$ might use different pathways to activate this σ^E-dependent transcriptional response.

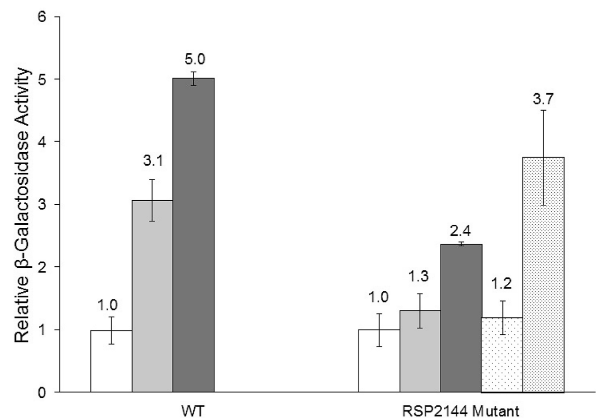

FIG 1 Activation of σ^E-dependent transcriptional responses in wild-type (WT) and ΔRSP2144 cells. Shown are relative β-galactosidase levels from a σ^E-dependent *rpoE*::*lacZ* fusion in cells before (white bars) and 2 h after exposure to $^1O_2$. $^1O_2$ was generated either by illuminating aerobically grown cells in the presence of oxygen and the photosensitizer methylene blue (light-gray bars) or by exposing photosynthetic cells to 30% O_2 in the light (dark-gray bars). For the ΔRSP2144 mutant, the LacZ levels from the same σ^E-dependent *rpoE*::*lacZ* fusion are shown 2 h after exposure to $^1O_2$ (exposure of aerobic cells to light and methylene blue) in cells harboring a plasmid lacking (stippled bars) or containing (checkerboard bars) the RSP2144 gene. For wild-type and ΔRSP2144 cells, a relative β-galactosidase level of 1.0 is equivalent to 75 and 95 units, respectively.

RESULTS

Some members of the direct σ^E regulon are needed for $^1O_2$ to fully activate the σ^E transcriptional response to this ROS. *R. sphaeroides* σ^E is a member of the group IV family of alternative σ factors (2, 3, 16). In some cases, members of the group IV σ factor regulon are needed to fully activate the transcriptional response (17). To test the role of genes transcribed by σ^E in the transcriptional response to $^1O_2$, we compared the abilities of this ROS to stimulate transcription from a known σ^E-dependent *lacZ* reporter gene (18, 19) in wild-type and mutant cells lacking individual regulon members. In wild-type cells, we observed a 3- to 5-fold increase in activity from a σ^E-dependent *lacZ* reporter gene within 2 h after the generation of $^1O_2$ (Fig. 1), by using either the photosensitizer methylene blue (exposing aerobic cells to light and methylene blue) or photopigments in the photosynthetic apparatus (exposing photosynthetic cultures to light and O_2) as a source of this ROS.

We found that mutations inactivating individual genes within the core σ^E regulon cause a defect in the activation of the transcriptional response to $^1O_2$, in contrast to the situation in wild-type cells. For example, cells lacking RSP2144 had wild-type levels of activity from the σ^E-dependent *lacZ* reporter gene in the absence of $^1O_2$, but they showed an increase in LacZ levels of less than 50% 2 h after $^1O_2$ was generated by exposing aerobic cells to light and methylene blue (Fig. 1). We know that the presence of carotenoids or an active σ^E protein is required for viability in the presence of $^1O_2$ (18). We also know that producing $^1O_2$ by exposing aerobically grown ΔRSP2144 cells (which have low levels of carotenoids) to methylene blue in the presence of light for multiple hours inhibits growth and ultimately leads to a decrease in viability (data not shown). To test if failure to fully activate transcription of the σ^E-dependent *lacZ* reporter gene was due to an indirect effect of cell growth or viability, we analyzed the activation of σ^E by $^1O_2$ in RSP2144 mutant cells grown photosynthetically and thus containing high levels of carotenoids. We know that carotenoid-containing mutants, including those lacking either σ^E (18) or RSP2144 (data not shown), are unable to activate the σ^E-dependent transcriptional response to $^1O_2$ but are viable and continue growing in the presence of this ROS. When light-excited photopigments from photosynthetic cells (which contain high levels of carotenoids) were used as a source of $^1O_2$, we found that transcription from the σ^E-dependent *lacZ* reporter gene was increased ~5-fold in wild-type cells but by only about half as much (2.4-fold) in cells lacking RSP2144 (Fig. 1). We also found that placing a copy of RSP2144 under the control of an IPTG (isopropyl-β-D-galactopyranoside)-inducible promoter complemented the defect in activation of the transcription of this σ^E-dependent reporter gene (Fig. 1), providing additional support for the notion that this activation defect was due to loss of this previously uncharacterized gene.

In addition, we found that cells containing an in-frame deletion of RSP1091, the first gene of the RSP1091-RSP1087 operon, had levels of activity from the σ^E-dependent *lacZ* reporter gene in the absence of $^1O_2$ that were comparable to that in wild-type cells (Fig. 2). However, this strain, like the RSP2144 mutant strain, also had a defect in the activation of this σ^E-dependent *lacZ* reporter gene 2 h after cells were exposed to $^1O_2$ (Fig. 2). In the absence of RSP1091, we observed an ~50% induction of the σ^E-dependent *lacZ* gene when $^1O_2$ was generated under aerobic conditions using the photosensitizer methylene blue (low carotenoid levels) and an ~3-fold increase in LacZ levels 2 h after shifting photosynthetic cells to aerobic conditions in the light (in which cells contain high carotenoid levels and generate $^1O_2$ from photochemistry). A similar defect in activation of the σ^E-dependent *lacZ* reporter gene activity in the presence of $^1O_2$ was observed in a strain containing an in-frame deletion of both RSP1091 and RSP1090 (data not

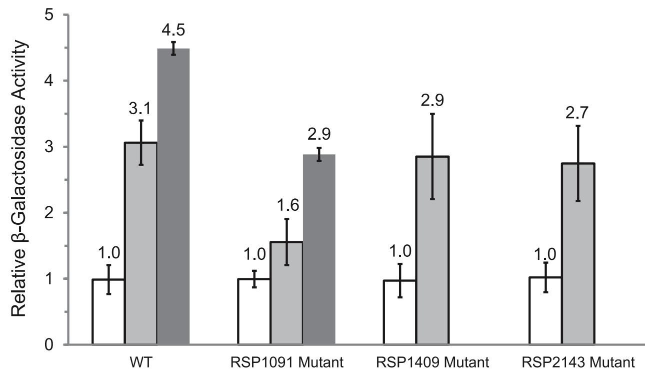

FIG 2 Activation of σ^E-dependent transcriptional responses in wild-type and mutant cells. Shown are relative β-galactosidase levels from a σ^E-dependent *rpoE*::*lacZ* fusion in cells before (white bars) and 2 h after exposure of cells to $^1O_2$. $^1O_2$ was generated by illuminating aerobic wild-type cells in the presence of oxygen with the photosensitizer methylene blue (light-gray bars) or by exposing photosynthetic cells to 30% O_2 in the light (dark-gray bars). Shown are data from wild-type cells as well as mutants lacking RSP1091, RSP1409, or RSP2143. A relative β-galactosidase level of 1.0 is equivalent to 75, 89, 69, and 65 units for wild-type, ΔRSP1091, ΔRSP1409, and ΔRSP2143 cells, respectively.

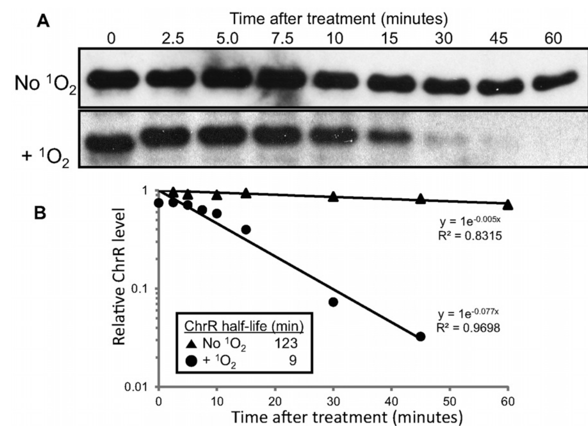

FIG 3 Stability of ChrR in the absence and presence of $^1O_2$. (A) Western blot analysis showing levels of ChrR as a function of time in the absence (top) and presence (bottom) of $^1O_2$ (generated by exposing cells to methylene blue, light, and O_2); (B) relative ChrR levels from the Western blots shown in panel A, used to calculate the indicated ChrR half-lives in the absence and presence of $^1O_2$ (~123 and 9 min, respectively).

shown). Thus, it is possible that some combination of RSP1091 and RSP1090 (see Discussion) is required for the full activation of σ^E activity in the presence of $^1O_2$ that is seen in wild-type cells.

We analyzed additional mutants to test the role of other members of the direct σ^E regulon in activating the transcriptional response to $^1O_2$. This analysis showed that wild-type cells and mutants lacking either RSP1409, RSP2143, or $RpoH_{II}$, each of which is a member of the extended σ^E regulon, showed similar levels of activity from this *lacZ* reporter gene in the absence $^1O_2$ and normal activation of this promoter 2 h after cells were exposed to this ROS (Fig. 2; data not shown for $RpoH_{II}$). Thus, we conclude that neither RSP1409, RSP2143, nor $RpoH_{II}$ is needed for normal activation of the σ^E-dependent transcriptional response in the presence of $^1O_2$. Rather, our data indicate that only a subset of the core members of the direct σ^E regulon genes are needed for full activation of this pathway in the presence of $^1O_2$.

ChrR degradation accompanies a $^1O_2$-dependent increase in σ^E activity. There are group IV sigma factors in which degradation of the anti-σ factor accompanies dissociation of the anti-σ–σ complex and subsequent activation of the transcriptional response; one such example is the *Escherichia coli* σ^E-RseA complex (16, 17). However, in the case of the group IV sigma factor *Streptomyces coelicolor* SigR, the oxidized form of its cognate anti-σ factor (RsrA), which is potentially generated in the presence of the inducing signal, is proposed to be recycled after it is reduced by thioredoxin (17, 20). To monitor the fate of ChrR when *R. sphaeroides* σ^E activity is increased, we analyzed the stability of this anti-σ factor when cells were and were not exposed to $^1O_2$. To do this, we inhibited new protein synthesis by treatment with chloramphenicol to prevent the synthesis of new ChrR, since we know that σ^E directly controls transcription of the *rpoE-chrR* operon (6, 19).

Western blot analysis using antibodies against ChrR indicated that levels of this protein remained relatively constant with time after chloramphenicol treatment of cells that were not exposed to $^1O_2$ (Fig. 3A). In contrast, ChrR levels decreased more rapidly, in a time-dependent manner, in cells that were exposed to $^1O_2$ (Fig. 3A). By measuring the time-dependent changes in ChrR levels, we calculated half-lives for this anti-σ factor of ~120 and ~10 min in the absence and presence of $^1O_2$, respectively (Fig. 3B). Thus, we conclude that exposure of cells to $^1O_2$ promotes ChrR degradation.

ChrR turnover is altered in mutants with activation defects in the σ^E-dependent response to $^1O_2$. The inability of selected mutants to fully activate transcription of σ^E-dependent target genes in the presence of $^1O_2$ prompted us to analyze ChrR turnover under these conditions. We found that cells lacking *rpoH$_{II}$*, a gene within the extended σ^E regulon, had a half-life for ChrR turnover in the presence of $^1O_2$ (10 min) (Fig. 4) comparable to that of wild-type cells (9 min) (Fig. 4). In contrast, the half-lives of ChrR turnover in the presence of $^1O_2$ in strains lacking either RSP2144, RSP1091, or both genes are reproducibly longer (16, 30, and 34 min, respectively) than observed in wild-type cells (9 min) (Fig. 4). In each of these activation-defective mutants, the half-life of ChrR is shorter than that observed in wild-type cells in the

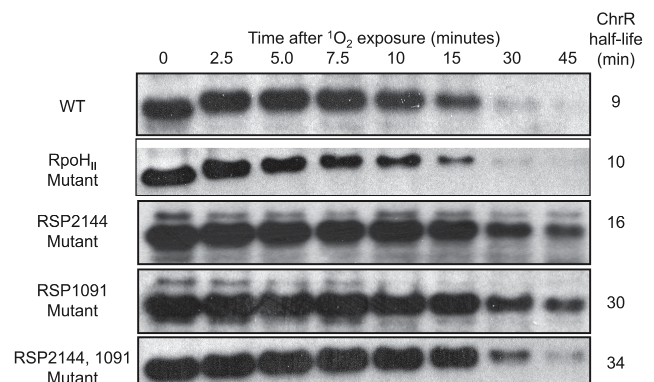

FIG 4 Stability of ChrR in wild-type and mutant cells in the presence of $^1O_2$. Western blot analysis showing ChrR levels as a function of time in the indicated strains. The column on the right shows the calculated ChrR half-lives in the presence of $^1O_2$ (generated by exposing cells to methylene blue, light, and O_2) in wild-type and mutant strains (calculated as described in the legend of Fig. 3).

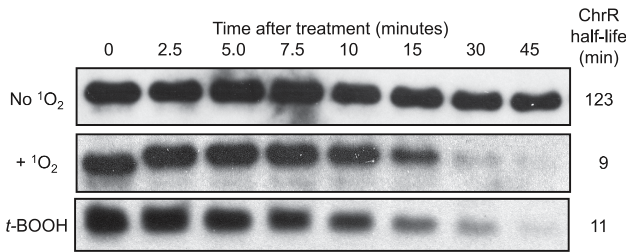

FIG 5 Stability of ChrR in wild-type cells in the absence or presence of $^1O_2$ or the organic hydroperoxide *t*-BOOH. Western blot analysis showing ChrR levels as a function of time. The column on the right shows the calculated ChrR half-life in the presence of $^1O_2$ (generated by exposing cells to methylene blue, light, and O_2) in wild-type and mutant strains (calculated as described in the legend of Fig. 3).

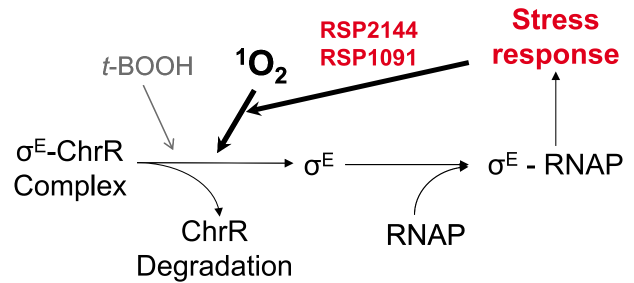

FIG 7 Model for activation of the σ^E-dependent stress response by $^1O_2$ and *t*-BOOH. Shown is the ability of $^1O_2$ and *t*-BOOH to promote ChrR degradation, releasing σ^E, so it binds RNA polymerase (RNAP) and activates the transcription of genes in the stress response. In red is shown the need for each of two σ^E target gene products (RSP2144 and RSP1091) to promote rapid ChrR degradation and the rapid degradation of this anti-σ factor in the absence of these two proteins when cells are exposed to *t*-BOOH (see the text).

absence of $^1O_2$ (120 min) (Fig. 3). However, the possible existence of multiple pathways to promote ChrR degradation (see below) and the known activation of the *rpoE-chrR* promoter by σ^E (18, 19) can explain why a reduction in the half-life of ChrR of this magnitude can produce the partial defect in σ^E activation in the presence of $^1O_2$ in cells lacking RSP2144 or RSP1091 (Fig. 1 and 2).

The organic hydroperoxide *t*-BOOH fully activates σ^E transcription in strains lacking genes needed for the normal response to $^1O_2$. Previous studies report that *tert*-butyl hydroperoxide (*t*-BOOH), an organic hydroperoxide, also increases σ^E activity in *R. sphaeroides* and *C. crescentus* (11, 15). However, it is possible that *t*-BOOH and $^1O_2$ promote dissociation of σ^E-ChrR complexes by different mechanisms (11). To test this hypothesis, we analyzed the induction of σ^E activity and the turnover of ChrR in response to *t*-BOOH in wild-type and mutant strains.

When we measured ChrR stability in wild-type cells treated with *t*-BOOH, we found that ChrR was degraded with a half-life of ~11 min, similar to that found in $^1O_2$-treated cells (~9 min), indicating that this organic hydroperoxide also promotes the turnover of this anti-σ factor (Fig. 5). The ability of *t*-BOOH to promote ChrR turnover explains why it activates expression of a σ^E-dependent reporter gene (11).

To test the role of direct σ^E regulon gene members in the activation of σ^E by *t*-BOOH, we analyzed the ability of this organic hydroperoxide to induce expression of the same σ^E-dependent *lacZ* reporter gene that we used before (Fig. 6). When we measured the activity of this σ^E-dependent reporter gene in wild-type cells and in mutant cells lacking RSP2144 or RSP1091, we found that levels of LacZ activity were comparable in all three strains before and 2 h after exposure to *t*-BOOH (Fig. 6). We also found that exposing these same wild-type and mutant cells to a combination of both *t*-BOOH and $^1O_2$ for 2 h at concentrations of these compounds that did not inhibit growth resulted in no further activation of this response (data not shown).

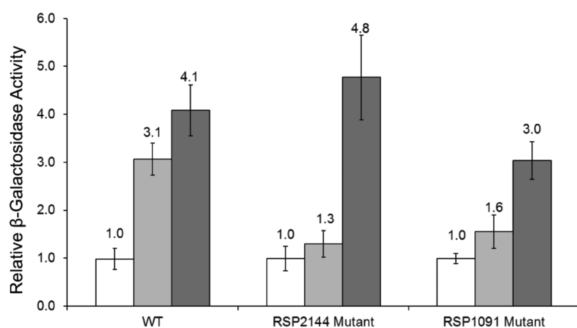

FIG 6 Activation of σ^E-dependent transcriptional responses in wild-type and mutant cells in the presence of the organic hydroperoxide *t*-BOOH. Shown are LacZ levels from a σ^E-dependent *rpoE::lacZ* fusion in wild-type cells or the indicated mutants before (white bars) and 2 h after exposure to $^1O_2$ (by exposing aerobic cells to methylene blue, light, and O_2) (light-gray bars) or *t*-BOOH (dark-gray bars).

From the above data, we conclude that *t*-BOOH activates the σ^E-dependent transcriptional response by promoting ChrR turnover even in the absence of proteins (RSP2144 and RSP1091) that are needed for the normal response to $^1O_2$ (ΔRSP2144 and RSP1091). A model to explain why $^1O_2$ and *t*-BOOH lead to ChrR turnover by different mechanisms is presented in the Discussion.

DISCUSSION

In this study, we analyzed how cells activate a transcriptional response to the ROS $^1O_2$. We found that $^1O_2$ stimulates turnover of the ChrR protein, an anti-σ factor which forms a complex with σ^E and prevents this sigma factor from binding RNA polymerase to activate gene expression in the absence of an inducing signal (19). Thus, we conclude that $^1O_2$ activates this response by promoting the turnover of ChrR, releasing σ^E so that it can bind RNA polymerase and recognize target genes.

We further found that mutants lacking a subset of genes within a previously described core σ^E regulon that is conserved across diverse bacteria (9) have defects both in stimulating ChrR turnover and in normal activation of this transcriptional response (Fig. 7). This activation defect appears to be specific to a subset of target genes, since strains lacking other members of the direct σ^E regulon (RSP2143 or RSP1409) exhibit normal rapid turnover of ChrR and full induction of this transcriptional response in the presence of $^1O_2$. From this, we conclude that several members of the core σ^E regulon play a previously unrealized regulatory role in the activation of the σ^E-dependent transcriptional response to $^1O_2$.

In addition, we found that activation of the σ^E-dependent transcriptional response is accompanied by ChrR turnover in the presence of two chemically different oxidants ($^1O_2$ and *t*-BOOH). However, we found that the organic hydroperoxide *t*-BOOH was able to activate the σ^E-dependent transcriptional response in mutants that exhibit activation defects in the presence of $^1O_2$ (Fig. 7).

From this, we conclude that $^1O_2$ and *t*-BOOH promote ChrR turnover by different mechanisms. Below, we place these observations in context, provide an explanation for our findings, and propose future experiments to answer questions posed by these new results.

Gene products needed for $^1O_2$ to activate the σ^E-dependent transcriptional response. One protein needed to activate the σ^E-dependent transcriptional response to $^1O_2$ is RSP2144, which is predicted to encode a homolog of an *S*-adenosylmethionine-dependent enzyme that produces cyclopropane fatty acids (2, 9, 18). The failure of ΔRSP2144 cells to activate σ^E activity in the presence of $^1O_2$ predicts that a result of its activity is needed to promote the rapid ChrR degradation seen when wild-type cells are exposed to singlet oxygen. Experiments are in progress to determine the function and to identify the product of RSP2144 in order to understand how loss of this protein produces a defect in ChrR turnover that alters the σ^E-dependent transcriptional response to $^1O_2$.

Proteins of unknown function encoded by the RSP1091-RSP1087 operon (2, 9) are also needed to promote the rapid ChrR turnover seen in wild-type cells and to fully activate the σ^E-dependent transcriptional response to $^1O_2$. A similar defect in activation of the σ^E-dependent transcriptional response to $^1O_2$ is observed in cells containing either a polar insertion in RSP1091 or an in-frame deletion in RSP1091, or both RSP1091 and RSP1090 (data not shown), assigning this defect to at least one uncharacterized protein encoded by this operon. Neither RSP1091 nor RSP1090 is a homologue of known proteases (2, 9), so it is not known if they are directly involved in ChrR turnover or needed to activate a pathway that targets this protein for degradation in the presence of $^1O_2$. To date, we have been unable to isolate strains containing only an in-frame deletion of RSP1090 in wild-type cells (E. C. Ziegelhoffer, unpublished), suggesting that this protein may also be needed for viability in the absence of $^1O_2$.

How might cells promote ChrR turnover? It was previously shown that $^1O_2$ and *t*-BOOH each promote dissociation of σ^E-ChrR complexes and that the ChrR C-terminal domain (CTD) is needed for this process (11). Consistent with this previous proposal, the current study found that *t*-BOOH leads to rapid ChrR turnover and full induction of σ^E activity in the RSP2144 and RSP1091 mutants that exhibit a defect in activation by $^1O_2$. However, the results in this paper also show that proteins needed for rapid ChrR turnover and full activation of σ^E activity in the presence of $^1O_2$ are not needed for these events when cells are exposed to *t*-BOOH (Fig. 7). This observation indicates that different proteins and possibly different molecular events are needed to initiate ChrR turnover and activate σ^E activity in the presence of $^1O_2$ and *t*-BOOH.

From the reported effects of oxidants such as *t*-BOOH on other proteins (1, 20), cysteine side chains (two of which are conserved in the ChrR CTD) are likely targets for modification by this compound. The ability of *t*-BOOH to promote ChrR turnover in the absence of proteins needed for activation by $^1O_2$ might also explain why mutants lacking proteins such as RSP2144 or RSP1091 produce only partial defects in activation of the σ^E-dependent transcriptional response to this ROS (see below). Experiments are in progress to determine how $^1O_2$ and *t*-BOOH each promote ChrR turnover and to define elements of the ChrR CTD needed to promote the degradation of this anti-σ factor in the presence of each compound.

Why might ChrR degradation be stimulated by both $^1O_2$ and *t*-BOOH? Our data predict that ChrR degradation is needed for cells to increase σ^E activity in the presence of two different oxidants, $^1O_2$ and *t*-BOOH. We propose that the co-occurrence of these oxidants in nature can explain why these two compounds activate this response (21). We know that σ^E activity is increased by $^1O_2$, a ROS that is formed by energy transfer to oxygen, but not by other types of ROS that are produced by electron transfer reactions (superoxide, hydrogen peroxide, or hydroxy radicals) (18). Thus, we propose that $^1O_2$ is a relevant activator of σ^E activity in cells which are exposed to this ROS as it is generated from either endogenous or exogenous processes (photochemistry in phototrophic organisms, photosensitizing, or enzyme-mediated reactions in both phototrophs and nonphotosynthetic cells).

Given the chemical reactivity of $^1O_2$, it is likely that organic hydroperoxides are produced at significant levels from both lipid peroxidation and pigment photooxidation when phototrophs encounter this ROS. Thus, we propose that the turnover of ChrR in the presence of either $^1O_2$ or organic hydroperoxides like *t*-BOOH allows two chemically unrelated but potentially associated stimuli to activate the σ^E-dependent transcriptional response (21, 22). If this is the case, the production of organic hydroperoxides when cells are exposed to $^1O_2$ can explain why we observe ChrR turnover and partial activation of σ^E-dependent transcription in cells lacking proteins needed for the response to $^1O_2$ (Fig. 7).

It is known that $^1O_2$ has a short half-life in cells due to its high reactivity (5, 6). Thus, we propose that the ability of both $^1O_2$ and *t*-BOOH to promote ChrR turnover allows for persistence of the σ^E-dependent transcriptional response after this stimulus ($^1O_2$) is gone. The use of ChrR as a master regulator of a crucial stress response (Fig. 7) also explains why several target genes that are activated either directly by σ^E or by other members of this transcriptional cascade have been linked to the survival of other cells in the presence of organic hydroperoxides (2, 9, 23).

Potential conservation of the $^1O_2$-dependent activation pathway. To date, σ^E-ChrR homologues have been identified in both photosynthetic and nonphotosynthetic members of the alpha- and gammaproteobacteria (3, 9). It is possible that the broad distribution of these master regulators and the high degree of conservation of selected residues in the ChrR CTD (3, 11) reflect the ability of either $^1O_2$ or organic hydroperoxides or both oxidants to promote the turnover of this protein. However, until more is known about the molecular mechanisms for activation of the transcriptional response by $^1O_2$ and *t*-BOOH, it is possible that some ChrR homologues respond only to a single oxidant or to another signal that is physiologically relevant in nature.

In sum, our studies have shed new light on several aspects of a conserved transcriptional response to the ROS $^1O_2$. First, we showed that several previously uncharacterized genes are required for full activation of the transcriptional response to $^1O_2$ by promoting the rapid turnover of ChrR, a negative regulator of σ^E activity. None of these proteins have significant amino acid sequence similarity to known proteases, so it is not yet clear how they act to signal or promote the rapid degradation of the ChrR that normally accompanies the activation of the σ^E-dependent transcriptional response to $^1O_2$. The observation that none of these newly identified regulators of the σ^E-dependent transcriptional response to $^1O_2$ are needed for ChrR turnover or activation of this pathway in the presence of the organic hydroperoxide *t*-BOOH suggests that these oxidants use different processes to

TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Description[a]	Source
Strains		
R. sphaeroides		
2.4.1	Wild-type strain	Lab strain
TF18	Δ*rpoE-chrR* mutation in 2.4.1	30
ΔChrR mutant	Δ*chrR* mutation in 2.4.1	19
ΔRSP1409 mutant	ΩSp^r insertion in RSP1409 coding sequence in 2.4.1	This work
ΔRSP2144 mutant	ΩSp^r insertion in RSP2144 coding sequence in 2.4.1	This work
ΔRSP1091 mutant	In-frame deletion in RSP1091 coding sequence in 2.4.1	This work
ΔRSP1091-RSP1090 mutant	In-frame deletion of both RSP1091 and RSP1090 coding sequences in 2.4.1	This work
ΔRSP2144 ΔRSP1091 mutant	In-frame deletion of both RSP2144 and RSP1091 coding sequences in 2.4.1	This work
ΩRSP1091 mutant	ΩSp^r insertion in RSP1091 coding sequence in 2.4.1	This work
TWNR01	2.4.1 (*rpoEp*::*lacZ*)	This work
TWNR02	ΔRSP2144 (*rpoEp*::*lacZ*)	This work
TWNR03	ΔRSP1091 (*rpoEp*::*lacZ*)	This work
E. coli		
DH5α	*supE44 lacU169* (ϕ80 Δ*lacZ*M15) *hsdR178 recA1 endA1 gyrA96 thi-1 relA1*	31
S17-1	C600::RP-4 2-(Tc::Mu) (Kn::Tn7) *thi pro hsdR* HsdM$^+$ *recA*	32
Plasmids		
pBlueScriptII KS−	Cloning vector; Ap^r	Agilent Technologies
pSUP202	Mobilizable suicide plasmid	32
pK18mobsacB	*oriV oriT mob sacB* Kn^r	26
pIND4	Expression vector inducible by IPTG; Kn^r	29
pIND5	NcoI site in pIND4 replaced by an NdeI site; Kn^r	This work
pJDN30	−39 to +17 *rpoEp*$_1$ promoter fragment fused with the *lacZ* reporter; Kn^r	19
pRKK81	Promoterless *lacZ* in pUC19*spf*; Ap^r	25
pND02	*lacZ-spf* fragment from pRKK81 (~3.4-kb HindIII-EcoRI fragment) in pK18mobsacB; Kn^r	This work
pND03	−85 to +630 *rpoE* fragment (EcoRI-NdeI) to EcoRI-AseI site in pND02; Kn^r	This work
pND04	877-bp PstI-StuI fragment of RSP1091-*rpoE* (to +87 of *rpoE*) in pND03; Kn^r	This work
pND05	562-bp SalI-StuI fragment of RSP1091-*rpoE* (to +87 of *rpoE*) in pND03; Kn^r	This work
pRSBY1	6.7-kb fragment containing RSP1091-RSP1087 (775 bp upstream to 1,357 bp downstream)	This work
pRSBY4	6.9-kb fragment containing RSP2143-RSP2144 (1.4 kb upstream to 2.8 kb downstream cloned into XbaI/KpnI digested pBlueScriptII (pBSII))	This work
pRS2144	1.7-kb fragment from pRSBY4 containing RSP2144 (207 bp upstream to 298 bp downstream) cloned into BamHI/ HindIII-digested pBSII	This work
pΔΩRSP2144-PstI	2.1-kb Sp^r cassette cloned into a filled-in PstI-digested pRS2144, deletes codon 259–874	This work
pΔΩ2144-202	3.5-kb filled-in XbaI-XhoI fragment from pΔΩRSP2144-PstI cloned into ScaI-digested pSUP202	This work
pRSBY2-R	2.4-kb fragment containing RSP1409 (1,066 bp upstream to 898 bp downstream) cloned into XbaI/KpnI-digested pBSII	This work
pBY2-RΩB	Deletion of 310 bp of RSP1409 in pRSBY2-R and insertion of ΩSp^r cassette	This work
pBY2-RΩB-F	4.4-kb PCR product from pBY2-RΩB cloned into ScaI-digested pSUP202	This work
pND11	RSP1091 in pIND5	This work
pND12	RSP1090 in pIND5	This work
pND13	RSP1091-RSP1090 in pIND5	This work

[a] Ap^r, ampicillin resistance.

activate this pathway (Fig. 7). In addition, we propose that the ability of both oxidants to promote ChrR degradation reflects an evolutionary adaptation to the formation of organic hydroperoxides when $^1O_2$ is generated. Experiments are in progress to use the information derived from these studies to further understand how cells activate this conserved transcriptional response to $^1O_2$.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *R. sphaeroides* strains (Table 1) were grown at 30°C in Sistrom's succinate-based minimal medium A (24). *E. coli* strains were grown at 37°C in Luria-Bertani medium. When necessary, media were supplemented with 25 μg/ml kanamycin to maintain plasmids. Five-hundred-milliliter *R. sphaeroides* cultures were grown aerobically by bubbling them with a mixture of 69% N_2, 30% O_2, and 1% CO_2 in the dark (18, 25). For photosynthetic growth, cultures were bubbled with a mixture of 95% N_2 and 5% CO_2 in front of an incandescent light source with an intensity of 10 W/m^2 (18, 25).

To generate $^1O_2$, aerobic *R. sphaeroides* cultures were exposed to methylene blue (0.1 μM to 1 μM) in the light (10 W/m^2) (18). For photosynthetically grown cells, cultures were switched to aerobic growth conditions with a light intensity of 10 W/m^2 (18, 25). To test the effect of

t-BOOH, final concentrations of 50 to 100 μM were added to aerobic cultures (11).

Construction of strains containing genomic *rpoE-lacZ* fusions. To construct strains containing a chromosomal insertion of an *rpoEp*::*lacZ* operon fusion, we first cloned a promoterless *lacZ* fragment (HindIII-EcoRI) from pRKK81 (25) into plasmid pK18mobsacB (26). Then, a 715-bp oligonucleotide containing most of the *rpoE* gene plus 85 bp of the *rpoE* upstream region was incorporated downstream of *lacZ* and the *spf* terminator. To this plasmid, an 887-bp DNA fragment containing part of the wild-type RSP1091 gene and the *rpoE* upstream region (to +87) was inserted upstream of the *lacZ* gene. The resulting plasmid (pND04), containing two identical *rpoE* promoter regions, attached to both ends of *lacZ*, was conjugated into wild-type cells. Similarly, to create the analogous *rpoEp*::*lacZ* construct for ΔRSP1091 or ΔRSP1091-RSP1090 cells, template DNA from ΔRSP1091 mutant cells was used as the source of the RSP1091 gene plus the *rpoE* promoter section of the plasmid construct. The resulting plasmid (pND05) was conjugated into ΔRSP1091 or ΔRSP1091-RSP1090 mutant cells. After selection for double crossovers in cells containing the *sacB* gene (26), colonies showing blue color on plates containing *t*-BOOH were selected and their genomic DNA was sequenced to confirm the incorporation of the *lacZ* fusion at the *rpoE-chrR* locus before further study.

Creation of an *R. sphaeroides* ΔRSP2144 strain. A fragment containing RSP2143-RSP2144 was amplified from genomic DNA and cloned into the XbaI and KpnI sites of pBlueScriptII (pBSII) to create plasmid pRSBY4 (Table 1), and the DNA sequence of the cloned genes was verified. To create pRS2144, RSP2144 was inserted as a 2.8-kb RSP2144-containing PCR fragment from pRSBY4 into the BamHI and HindIII sites of pBSII and the DNA sequence of RSP2144 in pRS2144 was verified. To create the ΔRSP2144 strain, a 608-bp PstI-PstI restriction fragment from pRS2144 was deleted, followed by insertion of an omega cartridge (27) that encodes spectinomycin resistance (SP^r) (ΩSp^r), resulting in a 203-amino-acid deletion from codon 259 through codon 874 in the middle of the RSP2144 gene. The resulting 3.5-kb DNA fragment was digested with XbaI and XhoI, filled in with the large Klenow fragment (Promega, Madison, WI), and cloned into the ScaI site of pSUP202 (Table 1). The insertion of this interrupted RSP2144 gene in the resulting 9.7-kb plasmid, pΩ2144-202, was confirmed by sequencing the RSP2144-ΩSp^r region before it was transformed into *E. coli* S17-1 for conjugation into *R. sphaeroides*. Sp^r colonies were screened for a tetracycline-sensitive (Tc^s) phenotype to identify strains in which the wild-type RSP2144 gene was replaced with the RSP2144::ΩSp^r gene. DNA from candidate colonies was screened by PCR to confirm the presence of the desired mutation and the absence of the wild-type RSP2144 gene.

Creation of *R. sphaeroides* RSP1091-RSP1087 operon mutant strains. A 6.6-kb fragment containing the RSP1091-RSP1087 operon plus flanking sequence was amplified from genomic *R. sphaeroides* 2.4.1 DNA and cloned into the XbaI and KpnI sites of pBSII to create plasmid pRSBY1 (Table 1), and the DNA sequence of the cloned genes was verified. To create a polar insertion disrupting the putative RSP1091-RSP1087 operon, an ΩSp^r cartridge from pHP45Ω was inserted into a unique SalI site, interrupting the RSP1091 gene at its 61st codon. The resulting plasmid was digested with Acc65I and AflI to excise the 5′ end of the operon plus the ΩSp^r insertion, blunt ended with the Klenow fragment of DNA polymerase, and cloned into ScaI-digested pSUP202. The resulting plasmid was transformed into *E. coli* S17-1 for conjugation into *R. sphaeroides*. Sp^r colonies were screened for a Tc^s phenotype to identify strains in which the wild-type RSP1091 gene was replaced with the RSP1091::ΩSp^r gene. Chromosomal DNA from candidates was isolated and used in PCRs to confirm the presence of the desired mutation and the absence of the wild-type RSP1091 gene.

To create the ΔRSP1091 mutant, pRSBY1 was first digested with EcoRI and AflII to remove downstream genes and improve cloning options. The DNA was then blunted with the Klenow fragment and allowed to religate. This construct was then digested with HincII and AfeI at sites uniquely located within RPS1091, resulting in an in-frame deletion of 351 of the 430 amino acids comprising RSP1091 when the remaining DNA was religated. This plasmid was then cut with Acc65I and XbaI, and the fragment was blunted with the Klenow fragment and inserted into the SmaI site of pK18mobsacB. After transformation into *E. coli* S17-1, the plasmid containing the ΔRSP1091 mutation was conjugated into *R. sphaeroides*, with selection for kanamycin resistance (Kn^r) encoded by pK18mobsacB. We screened DNA from sucrose-resistant cells for those lacking the pK18mobsacB plasmid and containing the ΔRSP1091 mutation described above using a combination of Kan^r screening, PCR, and DNA sequencing with specific primers.

To create the ΔRSP1090 mutant, pRSBY1 was digested with AflII and BspDI and blunted with the Klenow fragment, resulting in in-frame deletion of the central 196 amino acids of RSP1090 after religation of this plasmid, which was confirmed by DNA sequencing. This plasmid was digested with Acc65I and XbaI, and the resultant fragment was blunted with Klenow and inserted into the SmaI site of pK18mobsacB. After transformation into E. coli S17-1 cells, the plasmid was conjugated into *R. sphaeroides*, using screening for Kan^r and sucrose resistance, PCR, and DNA sequencing to identify strains containing the ΔRSP1090 mutation.

To create the ΔRSP1091-RSP1090 double mutant, a BsmI-Acc65I fragment from pΔ1091 was cloned into pΔ1090 to combine the two in-frame deletions in the RSP1091-RSP1087 operon. The resulting double-deletion plasmid was cut with Acc65I and HincII, and the fragment was blunted with Klenow and inserted into the SmaI site of pK18mobsacB. After transformation into E. coli S17-1 cells, the plasmid was conjugated into *R. sphaeroides*, using screening for Kan^r and sucrose resistance, PCR, and DNA sequencing to identify strains containing the RSP1090 mutation.

To create the ΔRSP2144 ΔRSP1091 double mutant, pΔΩ2144-202 was conjugated into the 2.4.1 ΔRSP1091 strain. Sp^r colonies were screened for a Tc^s phenotype to identify strains in which the wild-type RSP2144 gene was replaced with the RSP2144::ΩSp^r gene. Chromosomal DNA from candidates was isolated and used in PCRs to confirm the presence of the desired mutations (both ΔRSP2144 and ΔRSP1091) and the absence of the wild-type RSP2144 and RSP1091 genes.

Creation of a ΔRSP1409 *R. sphaeroides* strain. A fragment containing RSP1409 was amplified from genomic DNA and cloned into the XbaI and KpnI sites of pBlueScriptII (pBSII) to create pRSBY2-R (Table 1), and the sequence of RSP1409 was confirmed. The ΔRSP1409 strain was created by a method similar to that used previously (28). Plasmid pRSBY2-RΩB was generated by inserting an ΩSp^r cartridge into a 310-bp PCR-generated deletion in the RSP1409 coding sequence. To generate this deletion, we created primers that amplified the entire plasmid except for the deleted region. This mutant RSP1409 gene was cloned into the ScaI site of pSUP202 (Table 1). The resulting 12.2-kb plasmid (pRSBY2-RΩB-F) was sequenced to ensure the presence of the ΩSp^r cartridge in RSP1409. The plasmid was transformed into *E. coli* S17-1 for conjugation into *R. sphaeroides*. Sp^r colonies were screened for a Tc^s phenotype to identify strains in which the wild-type RSP1409 gene was replaced with the mutant allele; candidates were screened by PCR to confirm the presence of the mutant RSP1409 gene and the absence of the wild-type gene.

Ectopic expression of RSP2144 in *R. sphaeroides*. Plasmid pIND5 differs from pIND4 (29) only in the exchange of the NcoI restriction site for an NdeI restriction site. pIND5 was created by annealing two primers encoding the restriction recognition site change, digesting them with XbaI/HindIII, and ligating them into XbaI- and HindIII-digested pIND4. The restriction site alteration was verified by sequencing.

***β*-Galactosidase assays.** Previously, we used the differential rate of β-galactosidase synthesis to determine the σ^E activity of *R. sphaeroides* cells (18). However, the growth of RSP2144 and RSP1091 mutant cells was attenuated at longer time points after cells were exposed to $^1O_2$ (R. A. S. Lemke, E. C. Ziegelhoffer, and T. J. Donohue, unpublished), so we could not always use the differential rates of β-galactosidase synthesis to measure promoter activity. Instead, we used an alternative calculation (M500)

for β-galactosidase activity by measuring enzyme activity (absorbance at 420 nm) and the densities of cultures at 500 nm, since methylene blue absorbs strongly between 609 and 668 nm. M500 values were calculated by dividing the A_{420} by the A_{500} as follows: $(A_{420} \times 1{,}000)/(A_{500} \times$ cell volume in assay [ml] $\times$ time of assay [min]) (18). Absorbances at 500 nm and 600 nm were in direct proportion for phototrophic or aerobically grown *R. sphaeroides* cells (data not shown).

Western blot experiments. Exponential-phase cells were treated with 200 μg/ml chloramphenicol to inhibit protein synthesis (11). Ice-cold trichloroacetic acid (10% final concentration) was added to ~5×10^8 exponential-phase cells, followed by centrifugation (13,000 × *g* for 10 min) to precipitate proteins. The supernatant was aspirated and the pellet washed twice with ice-cold 100% acetone. After evaporation of residual acetone, the cell pellet was suspended in sufficient 1× LDS loading dye (Invitrogen, Carlsbad, CA) to provide a sample containing material from ~2×10^6 cells/μl before 5 to 10 μl of sample was loaded onto an NuPAGE gel (Invitrogen, Carlsbad, CA). Western blotting used rabbit antibody against ChrR (11).

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